

Running head: *Vaterite in the avian eggshell*

A rare mineral, vaterite, acts as a shock absorber in the eggshell of a communally nesting bird

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Birds' eggshells are primarily composed of calcite, an abundant polymorph of calcium carbonate (CaCO_3). However, the eggshells of some species are coated with spherules of vaterite, a rare and thermodynamically unstable polymorph of CaCO_3 , the function of which remains unknown. Here we experimentally test the mechanical and physiological effects of the vaterite coating on eggshells of the Greater Ani *Crotophaga major*, a tropical cuckoo. Vaterite removal did not affect vapour conductance rates across the eggshell, indicating that the vaterite coating does not influence gas exchange during embryonic development. By contrast, nanoindentation revealed that the hardness and elasticity of vaterite is similar to that of calcite, and white light interferometry showed that the vaterite layer increased the total thickness of the shell cuticle by up to 10%. Furthermore, calculations of contact mechanics found that when two eggs come into contact, the depth of the surface deformation caused by the contact is far less than the thickness of the vaterite coating. These results suggest the layer of vaterite spherules may act as a 'shock absorber' for the underlying calcite shell, protecting it from mechanical damage caused by collision with other eggs in the nest and reducing the risk of eggshell fracture during incubation.

Keywords: calcite, *Crotophaga major*, Cuculidae, Hertzian mechanics, nanoindentation, Young's modulus

Calcium carbonate, the most abundant biomineral on Earth, is found in three anhydrous polymorphs: calcite, aragonite and vaterite, in order of decreasing thermodynamic stability. Calcite is the primary component of avian eggshells and the shells of many marine organisms, whereas aragonite is found in turtle eggshells, mollusc shells and corals. By contrast, vaterite is rare in nature, possibly due to its poor thermodynamic stability (Kabalak-Amitai *et al.* 2013). It is found in gallstones, renal calculi, and aberrant fish otoliths and mollusc shells (Tullett *et al.* 1976). Almost forty years ago, Board and Perrott (1979) found that the eggshells of a few bird species are coated with a thin, chalky layer of vaterite which overlays the calcite shell. This layer, formed by nanometer-scale spherules of vaterite, is confirmed to occur in only two groups of birds: waterbirds (Pelecaniformes: pelicans; Suliformes: gannets and anhingas), and communally breeding cuckoos (Cuculiformes, including the genera *Crotophaga* and *Guira*).

Several hypotheses have been proposed to explain the adaptive function of vaterite in the eggshell cuticle of these species. Board and Perrott (1979) originally posited that the vaterite layer might form an inorganic barrier that protects the porous shell, preventing microbial colonisation of the egg. In support of this hypothesis, a recent experimental study found that cuticular nanospheres of calcium phosphate do indeed reduce the risk of microbial colonisation, suggesting that vaterite might have a similar effect (D'Alba *et al.* 2014). Alternatively, Board and Perrott (1979) proposed that vaterite could act as a “shock absorber” for the underlying calcite shell, protecting it from mechanical damage caused by collision with other eggs in the nest and reducing the risk of eggshell fracture during incubation. Finally, Board and Scott (1980) found that vaterite spherules often cover the system of pore canals that allow gas exchange across the eggshell during embryonic development, suggesting that vaterite could alter the rate of water loss during incubation (either decreasing it, by blocking vapour conductance through the

pore; or increasing it, by altering the humidity gradient across the shell boundary). Neither the mechanical properties of vaterite nor its effects on embryonic respiration have been experimentally tested, so the latter two hypotheses have never been investigated.

In this study, we used eggshells of the Greater Ani *Crotophaga major*, a communally breeding cuckoo, to investigate whether the vaterite coating affects the mechanical and/or physiological properties of the calcite shell. The social system of this species provides an ideal opportunity to test these hypotheses, since communal breeding imposes unique physical challenges to the embryo during development. Greater Ani breeding groups contain up to four females, who lay their eggs into a single nest and cooperatively provide care to the communal clutch (Riehl & Jara 2009). Communal clutch sizes are large (up to 15 eggs per nest) and eggs collide with substantial force as the adults turn them during incubation, suggesting that selection has favoured adaptations that reduce the risk of shell fracture during incubation (Vehrencamp 1978, Riehl 2011). Furthermore, like most cuckoos, anis exhibit exceptionally rapid embryonic development (an eleven-day incubation period for a 30 g egg, Riehl & Jara 2009), suggesting high rates of water loss across the shell during incubation (Metcalf *et al.* 1981, Portugal *et al.* 2014). Finally, vaterite is known to play a role in visual signalling within ani nesting groups. Breeding adults use the appearance of the vaterite coating on the eggshell as a visual cue to judge the age of the egg: freshly laid eggs are coated with white vaterite, but this chalky coating is scratched during incubation to reveal the turquoise-blue calcite underneath (Riehl 2010a; Fig. 1).

In this study, we ask whether the vaterite coating of the ani eggshell also affects the properties of the eggshell itself, specifically whether it (1) alters the rate of gas exchange across the eggshell surface, influencing embryonic development by either increasing or decreasing the

rate of water loss during incubation; or (2) provides protection against physical damage to the calcite shell caused by contact between eggs, reducing the risk of fracture during incubation.

METHODS

Study species and eggshell samples

In July 2015 we collected freshly laid, un-incubated eggs of Greater Anis from a nesting population in the Barro Colorado Nature Monument, Panama (9° 09' 22.5" N, 79° 50' 55.4" W). Field work was registered and approved through the Smithsonian Tropical Research Institute, which operates the field station on Barro Colorado Island. Eggs were emptied of their contents and stored in dry conditions at room temperature. X-ray diffraction was first used to confirm the two polymorphs of calcium carbonate present in the shell. Spectra were acquired using a D8 Advance powder x-ray diffractometer (Bruker, UK) operating in reflection geometry mode. The diffractometer operates using a Cu x-ray source. Measurements were performed over the range $5^\circ < 2\theta < 100^\circ$ using a 0.02° step size. The time taken per step was 0.7 s, with a total measurement time of approximately 1 h.

Eggshell conductance: does vaterite alter rates of gas exchange?

We used a matched-pairs design to compare the rate of water vapour conductance across fragments of ani eggshells with and without the vaterite coating. We collected 20 eggs from different females across the study area and cut two shell fragments of 1 cm x 1 cm from the equator of each egg. The vaterite coating was left intact on one fragment and removed from the second fragment by moistening the shell's surface with a damp cotton swab (Fig. 1). This technique removes the vaterite spherules without occluding the shell pores: because vaterite is

more soluble and less thermodynamically stable than calcite, vaterite crystals dissolve upon prolonged contact with water whereas the insoluble calcite remains intact (Katsifaros & Spanos 1990). We then measured water vapour conductance from the 40 shell fragments, providing 20 matched pairs of samples from different eggs. One sample cracked during the measurement period and was excluded from analyses, so the final sample size was 19 matched pairs.

Water vapour conductance was measured using the protocols described in Portugal *et al.* (2010). Briefly, each conductance sampling unit consisted of a 0.5 mL individual PCR tube and a shell fragment. The units were prepared by removing the lids of the PCR tubes and filling each tube with 250 μ L of distilled water. A thin layer of clear silicone was then applied around the rim of each tube, and one shell fragment was glued over the top of each tube (with the inside of the shell facing down). The PCR tubes with fragments were placed in a rack and left to dry at room temperature (24 $^{\circ}$ C) for 24 h. Cyanoacrylate glue was then applied around the seal (on the underside of the fragment) to ensure an airtight join. A rack containing all 40 conductance units was placed inside a polystyrene desiccator cabinet with 100 g of silica desiccant in a room at standard ambient temperature and pressure (25 $^{\circ}$ C and 760 torr). Desiccant was replaced daily. Each unit was weighed upon initial placement in the desiccator to the nearest 0.0001 g with a Mettler Toledo analytical balance. Measurements were highly repeatable ($r = 0.99996$, determined by weighing five randomly chosen samples five times each). Each unit was weighed every 24 h (± 1 h) thereafter for three days, for a total of four measurements per unit. Mass loss from day one to day two were not included in the analyses since mass loss in the first 24 h may be higher as the shell itself dries out in the desiccator (Portugal *et al.* 2010). Therefore, the mass loss for each fragment was determined for two 24 h periods (Day 2-3 and Day 3-4) and these values were averaged to give one measurement of the mass of water lost through the eggshell

over each 24 h period (i.e. the vapour conductance, in mg/day). We used a matched-pairs *t*-test to compare conductance rates of samples with and without vaterite, since data were normally distributed and the variances around the two sample means were equal (2.03 mg/day).

Calculations were performed in Stata 14.1.

Contact mechanics: does vaterite act as a shock absorber?

We measured the hardness and Young's modulus (elasticity) of calcite and vaterite present in the ani eggshell using a Nanoindenter XP (MTS, USA) employing a diamond-coated Berkovich indenter. For each of eight eggs, 64 indentations were performed on separate calcite- and vaterite-presenting regions over an area of dimensions 70 μm x 70 μm . Samples were indented at a strain rate of 0.05 s^{-1} to a maximum depth of 500 nm. Young's moduli were calculated from analysis of the load-displacement data, fitting a second order polynomial to the unloading curve (Bufford *et al.* 2014). The Poisson's ratio of the calcium carbonate was assumed to be 0.3. Finally, the Hertzian theory of contact mechanics (Johnson 2008) was used to calculate the contact pressure for two eggs in contact under a compressive load of 35 g, the upper bound for the mass of a Greater Ani egg (Riehl 2010b), using the Young's modulus values measured by nanoindentation (details in Supplementary Methods). Data were analysed in Stata 14.1 and tested for normality with Shapiro-Wilk's test.

RESULTS

X-ray diffraction confirmed that two mineral phases of calcium carbonate were present in the acquired spectra, calcite and vaterite (Fig. S1). Images captured via scanning electron microscopy also revealed the structure of the vaterite nanospheres (Fig. 2). We obtained images

of the topography of the vaterite/calcite interface using white light interferometry, showing the mean thickness of the vaterite coating to be $18.5 \pm 8.5 \mu\text{m}$ ($n = 5$; range = $8.5 - 28.5 \mu\text{m}$; Fig. S2). Mean thickness of the calcite shell, measured with callipers, was $300 \pm \text{SD} = 30 \mu\text{m}$ ($N = 17$; range = $250 - 340 \mu\text{m}$).

We found no significant difference between rates of mass loss ($\text{mg}^{-1}/\text{day}^{-1}/\text{torr}^{-1}$) for shell fragments with and without vaterite (mean \pm SD = 0.21 ± 0.06 and 0.22 ± 0.06 , respectively; matched-pairs $t_{18} = -0.73$, $P = 0.47$; Fig. 3). Nanoindentation of eggshells revealed that the hardness and elasticity of vaterite is comparable to that of calcite: the Young's modulus (mean \pm SE) of vaterite and calcite were $E_{\text{vaterite}} = 4.00 \pm 0.44 \text{ GPa}$ and $E_{\text{calcite}} = 3.52 \pm 0.81 \text{ GPa}$, respectively (Fig. 4). This difference in elasticity was not statistically significant (Wilcoxon sign-rank test; $z = 0.56$, $P = 0.58$). The hardness of vaterite was also similar to that of calcite ($139.21 \pm 25.28 \text{ MPa}$ and $130.49 \pm 38.16 \text{ MPa}$, respectively; $z = 0.14$, $P = 0.88$; Fig. 4).

Using the measured values of Young's modulus (elasticity) for vaterite, the depth of the deformation caused by two eggs coming into contact was calculated to be approximately $1 \mu\text{m}$, assuming a compressive load of 35 g. This suggests that the thickness of the vaterite coating on an ani egg ($8\text{-}28 \mu\text{m}$) is far in excess of the depth of surface deformation caused by the contact.

DISCUSSION

Our experimental tests found that the vaterite coating of the ani eggshell does not increase rates of gas exchange, and that the mean conductance value of ani eggshells is $0.21 \pm 0.06 \text{ mg day}^{-1} \text{ torr}^{-1}$. This value is, if anything, lower than expected, given that anis exhibit rapid embryonic development and that eggs are laid in cup-shaped stick nests. The confined nest microclimate of cup nests typically leads to increased humidity, so a higher conductance (GH_2O) is necessary to

ensure a sufficient humidity gradient between the nest environment and the inside of the egg to allow optimal water loss during incubation (Portugal *et al.* 2014). In British birds, for example, the mean conductance value of species that build cup nests ($0.28 \pm 0.24 \text{ mg day}^{-1} \text{ torr}^{-1}$) was significantly higher than that of species that build shallow tree nests ($0.22 \pm 0.32 \text{ mg day}^{-1} \text{ torr}^{-1}$). The value that we obtained for ani eggshell conductance is remarkably similar to the latter figure, supporting recent findings that the eggs of cuckoos do not show higher rates of gas exchange despite exhibiting rapid embryonic development (Portugal *et al.* 2014).

In support of the hypothesis that the vaterite coating has evolved as an adaptation to cushion the egg from contact in large clutches, we found that the calcite layer of the ani eggshell is thicker than would be predicted by the mass of the egg. We obtained measurements of 250 – 340 μm for eggs of 27 – 32 g, whereas predicted thickness values for non-passerine eggshells of this mass fall between 240 and 255 μm (Ar *et al.* 1979). The layer of vaterite spherules further increases the thickness of the cuticle by up to 28 μm , consistent with earlier observations that ani eggshells seem unusually thick for their size (Vehrencamp 1978). Although the hardness and elasticity of calcite and vaterite-presenting regions of the shell were similar, there was substantial variation in both measurements for calcite and vaterite across individual eggs. This suggests that the composition of the vaterite and calcite layers may also vary across individual eggs, which could affect the risk of fracture during development. Egg mass is highly variable within and between females, suggesting that maternal body condition and maternal allocation of calcium to the shell structure may also be sources of variation (Riehl 2010b).

In the Pelecaniformes and Suliformes, the other lineages in which vaterite spherules have evolved, clutch sizes are not particularly large and it is not known whether the eggshells of these species are also under selection for increased thickness. Cooke (1979) noted that the vaterite

layer on the eggshells of Northern Gannets *Morus bassanus* tended to be much thinner when contaminated with the organochlorine DDE, and suggested that the vaterite layer was important in maintaining the overall strength and integrity of the eggshell. However, in Anhingas *Anhinga anhinga*, Colacino *et al.* (1985) found that the vaterite coating appeared to increase conductance across the shell boundary, supporting a physiological rather than mechanical function. It seems likely that vaterite serves more than one adaptive function across (and perhaps within) waterbird species, and more studies are needed in Pelecaniformes and Suliformes that simultaneously test these non-mutually exclusive hypotheses.

A final hypothesis which remains untested is that the vaterite nanospheres on the eggshell surface form a protective barrier against microbial infection (Board & Perrott 1979). Vaterite nanospheres appear to block the pore canals while still allowing respiratory gases to diffuse through the shell, potentially forming a mineralized barrier resistant to bacterial colonisation. In support of this hypothesis, D'Alba *et al.* (2014) found that cuticular nanospheres of hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) had a hydrophobic effect, reducing the likelihood of bacterial attachment on the eggshells of the Australian Brushturkey *Alectura lathami*. Furthermore, a subsequent study found that cuticular nanospheres occur most commonly on the eggs of species that nest in warm, humid environments, suggesting an antimicrobial function (D'Alba *et al.* 2016). These nanospheres are most commonly formed of hydroxyapatite or other forms of calcium phosphate (D'Alba *et al.* 2016), but vaterite may play a similar role.

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Figure 1. Eggs of the Greater Ani *Crotophaga major* showing (a) a recently laid egg with the white vaterite coating intact; (b) natural abrasion of the vaterite coating during incubation, revealing patches of the blue calcite shell underneath; and (c) artificial removal of the entire vaterite coating.

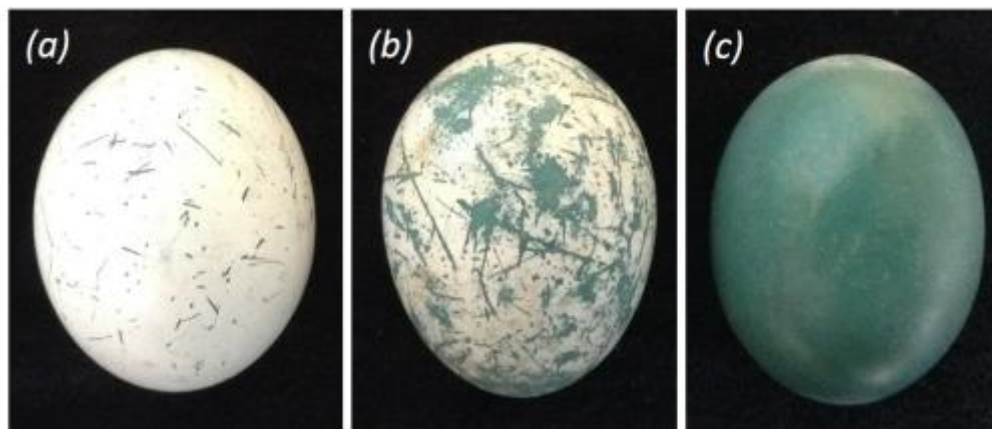


Figure 2. Scanning electron microscope (SEM) images of Greater Ani eggshells, showing (a) a shell with vaterite coating intact, revealing the spherical structure of vaterite nanospheres; and (b) a calcite shell with vaterite removed.

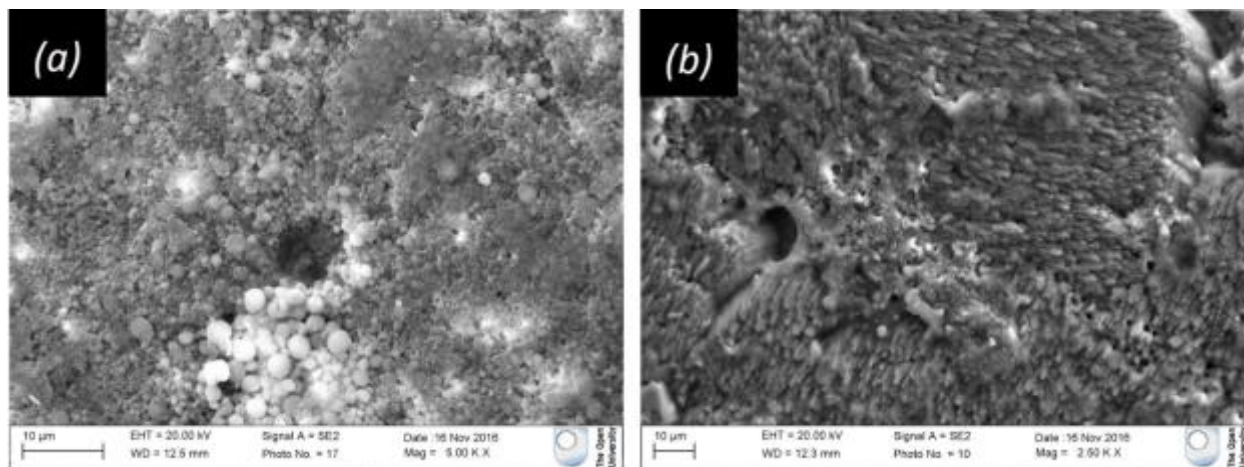


Figure 3. Matched-pairs measurements of vapour conductance (mass of water lost in g/day) for fragments from 19 Greater Ani eggshells (laid by 19 females) with the vaterite coating intact (open bars) or with the vaterite coating removed (calcite only; filled bars).

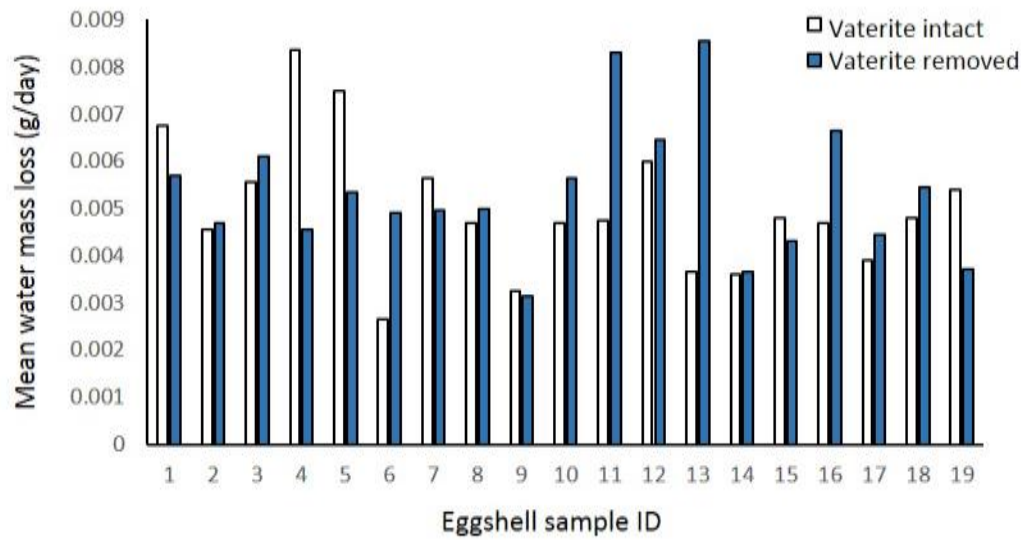


Figure 4. Nanoindentation measurements from eight eggshells laid by eight different female Greater Anis, showing (a) Young's modulus (GPa), and (b) hardness (MPa). Each bracket represents measurements from regions from the same egg presenting vaterite (open bars) or calcite (filled bars).

